*AN L-GLUTAMINE REQUIREMENT FOR INTERCELLULAR ADHESION**

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Abstract.—Intercellular adhesion presumably involves components of the cell surface, but the chemical nature of these substances is not known. The present studies suggest that complex carbohydrates are required for the adhesion of at least one type of animal cell. Single cells obtained from "embryoid bodies," the ascites-grown form of a mouse teratoma, aggregated in a complex tissue culture medium, but not in a glucose balanced salts solution. The active component of the tissue culture medium was identified as L-glutamine, and the only compounds found to replace it were the hexosamines D-glucosamine and D-mannosamine. A variety of studies indicated that the three compounds were active as a consequence of metabolic reactions. These results are consistent with known metabolic pathways and indicate that the conversion of nonadhesive to adhesive teratoma cells requires the synthesis of glycoproteins, glycolipids, and/or polysaccharides.

The adhesive properties of cells are apparently involved in phenomena such as morphogenesis, cell division, and the spread of tumor cells.[1–3] Extensive studies in several laboratories (see refs. 1–3) have defined the physical parameters and some protein "factors" that promote or retard adhesion,[1–6] but the nature of the cell-surface components involved in intercellular adhesion remains completely unknown. The system described in this report should facilitate identification of the substances required for adhesion of at least one type of animal cell.

Single cells obtained from "embryoid bodies," the ascites-grown form of a mouse teratoma, adhered to each other when maintained in a complex tissue culture medium (Medium 199), but were unable to aggregate in a glucose balanced salts solution. Medium 199 contains 51 components in addition to glucose and salts, but only one, L-glutamine, promoted aggregation when added to the glucose balanced salts solution; omission of L-glutamine from Medium 199 prevented aggregation. In addition, the rate of adhesion of the teratoma cells in glutamine-supplemented glucose balanced salts solution was comparable to that in complete Medium 199. Only two compounds were found to replace L-glutamine—the hexosamines D-glucosamine and D-mannosamine. These results suggest that L-glutamine is required for the synthesis of complex carbohydrates responsible for intercellular adhesion of the teratoma cells.

Materials and Methods.—Teratoma: An ascites subline (402AX) of strain 129 teratoma[7] was obtained in 1966 from Dr. Leroy Stevens of the Jackson Laboratory and has been maintained by intraperitoneal passage in 10–14-weeks-old 129/J male mice. In the ascites fluid, the tumor consists of rapidly proliferating cell aggregates called "embryoid bodies." These bodies contain two, or perhaps three, morphologically distinct cell types capable of differentiating into numerous cell types when implanted in tissues.[7]

Media: In the following and in all media containing supplements such as L-glutamine or the hexosamines, the pH was adjusted to 7.5 at 37° with 0.010 *M* Hepes buffer[8] in place of bicarbonate; Hepes (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid) was purchased from Calbiochem. Medium 199, the components of Medium 199, and Hanks' balanced salt solution were obtained from Grand Island Biological Co., Grand Island, N.Y. Hanks' balanced salt solution contains Na^+, K^+, Mg^{++}, Ca^{++}, Cl^-, HPO_4^{--}, SO_4^{--}, and glucose. Medium 199 contains 51 additional compounds including vitamins and cofactors, amino acids, purines, pyrimidines, nucleotides, etc. A modification of Moscona's calcium magnesium-free "saline" was used,[9] and contained the following (*M*): Hepes, 0.010; NaCl, 0.14; KCl, 0.004; NaH_2PO_4, 3.6×10^{-4}; KH_2PO_4, 1.8×10^{-4}; glucose, 0.011; pH 7.5 at 37° (CMF).

Materials: All materials were obtained from commercial sources;[10] the hexosamines and their N-acetyl derivatives were also prepared as described.[11, 12] The purity of the amino sugars and their N-acetyl derivatives was established by paper chromatography and electrophoresis.[13]

Preparation of single cell suspensions: The tumors were allowed to develop intraperitoneally in strain 129 mice for 12–22 days, the animals sacrificed by cervical dislocation, and the peritoneal contents rapidly transferred to Hanks' solution containing 2.5 units heparin/ml (heparin did not influence the glutamine response described below). After washing once with the same solution, 1.5 ml of packed embryoid bodies were incubated at 37° for 10 min in 15 ml of CMF, and treated for 20 min at 37° in 3 ml of CMF containing 21 mg of Difco 1:200 crude trypsin. The embryoids, which appeared intact, were washed with 30 ml of Hanks' balanced salt solution containing 10 μg DNase/ml, suspended in 10 ml of the same medium, and then dissociated by gentle aspiration (three times) through a 27-gauge 0.5-inch hypodermic syringe needle. The resulting suspension was diluted to 30 ml and centrifuged at 600 rpm in an International PR-2 model centrifuge (head no. 269) for 10 min at 5°; the pellet of single cells was gently suspended in 30 ml of Hanks' solution containing DNase, centrifuged, and washed once again. The single cells were then suspended in the medium in which aggregation was to be measured; all such media contained 5 μg DNase per ml.

Rate of aggregation: A convenient method for quantitatively evaluating the effects of chemical and physical agents on the early kinetics of cell aggregation has recently been developed in this laboratory. The procedure was used to study parameters affecting the rate of aggregation of 8–10-day embryonic chick neural retina cells and to assay a protein from horse serum that promoted adhesion of these cells.[5, 6] This method depends on determining the number of single cells remaining in a shaken suspension as a function of time, when the disappearance of single cells results from adhesion to each other. Essentially all of the experiments performed with neural retina cells[5] were repeated with the teratoma cells, making appropriate adjustment for the larger size of the latter (diameter, 15 ± 3 μ).

At Coulter Counter settings 1/amplification = 8, 1/aperture current = 0.707, and a window setting 10–100, approximately 80% of the single cells were included in the cell count, while aggregates, debris, and "leaky" cells were excluded. (Both the Coulter Counter and hemocytometer methods showed that single cells comprised 85–95% of the total cell population in the suspensions.) Assays were conducted with approximately $3.5–3.8 \times 10^5$ cells/ml, with 3 ml of suspension at pH 7.5 in 10 ml Micro-Fernbach flasks (Bellco no. 608) rotating at 76 rpm at 37°. The validity of the assay, i.e., that the disappearance of single teratoma cells represented aggregation and not, for example, lysis or loss of viability, was established as described.[5] Viability studies were also conducted on cells maintained at 37° in Hanks' solution as follows: (*a*) L-glutamine was added at various times, and the cells responded normally and aggregated at the usual rate (Fig. 2); (*b*) the cells were injected subcutaneously into 129/J mice and gave rise to tumors.

Embryonic neural retina cells aggregate rapidly at either 25° or 37°;[5] at the latter temperature, 50% of the single cells adhere to each other in 30 min. By contrast, the teratoma cells did not adhere to each other at 25° and aggregated relatively slowly at 37°;

the disappearance of 25–50% of the single cells required 5 to 6 hr in Medium 199. This fact was of concern, since attempts to study intercellular adhesion in simplified, and therefore deficient, media over prolonged periods of time could yield results where adhesion was effected as a secondary response to metabolic derangements within the cells. While many experiments were conducted for periods as long as 8 hr, and some results presented below involve 3- to 4-hr incubations, major emphasis was placed on the *initial* rate of aggregation of the teratoma cells, i.e., the rate over the first 60–90 min of incubation. During this time, about 10% of the cells aggregated. Despite the obvious experimental problems encountered in measuring small changes, the procedure gave reproducible results. Single cell densities were measured with duplicate or triplicate suspensions maintained in separate flasks; each value presented below is the mean of the replicates. Cell densities in each flask were obtained by counting at least three samples and taking the average. Replicate flasks gave values that differed from each other by an average of 2%; the range of duplicate values for one experiment is shown in Figure 3*B*. In addition, the results were reproducible from one experiment to another; for example, the L-glutamine requirement was observed with over 100 separate cell preparations.

Results.—Trypsin dissociation of neural retina, limb bud, and other embryonic tissues generally yields cells that reassociate rapidly in Hanks' balanced salt solution medium.[1–5] By contrast, the teratoma cells described in this report did not aggregate for at least 60–90 minutes in this medium (Fig. 1); in about half the preparations, slight aggregation was noted after two to three hours of incubation. Investigation of the failure of the teratoma cells to adhere to each other in Hanks' solution gave the following results (Figs. 1–4). (1) Teratoma cells did aggregate in Medium 199. (2) Only one constituent of Medium 199, L-gluta-

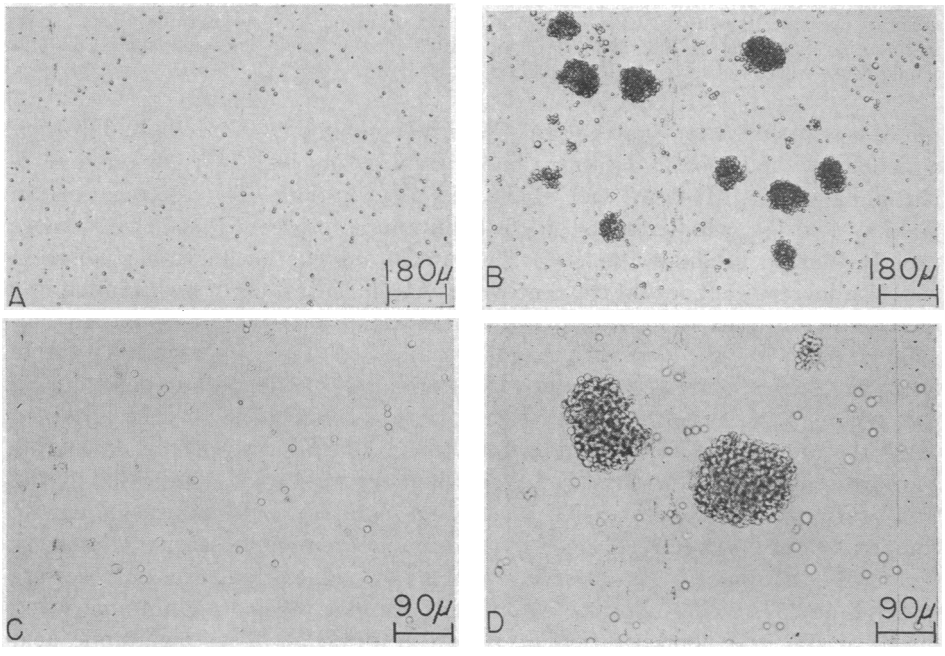

FIG. 1.—Effect of L-glutamine upon aggregation of teratoma cells. Cell suspensions were prepared and rotated for 200 min as described in the text; (*A* and *C*), Medium 199 minus L-glutamine; (*B* and *D*), Hanks' balanced salt solution supplemented with 3.5 mM L-glutamine.

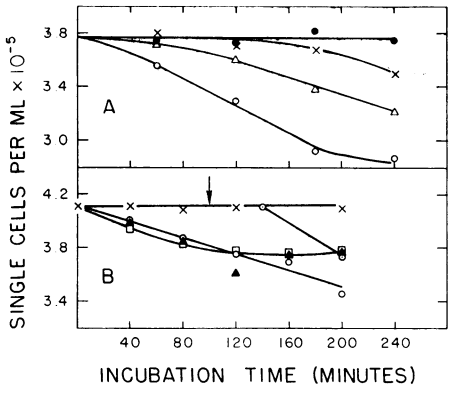

FIG. 2.—Effect of media upon aggregation of teratoma cells. The following media were tested with two different cell preparations, *A* and *B*, as described in the text: ●, Medium 199 lacking L-glutamine; x, Hanks' solution; △, Medium 199; ○, Hanks' solution containing 6.9 mM L-glutamine; ▲, Hanks' solution containing 6.9 mM D-glucosamine; □, Hanks' solution containing 6.9 mM D-mannosamine. The arrow in experiment *B* indicates the time of addition of L-glutamine to Hanks' solution to a final concentration of 6.9 mM (⊙). The variability observed with different cell preparations upon prolonged exposure to Hanks' solution is shown by *A* and *B* (see text). Single flasks were used for each point in *B*.

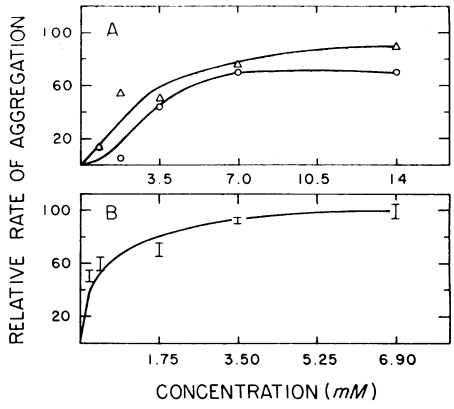

FIG. 3.—Effect of concentration of L-glutamine, D-glucosamine, and D-mannosamine upon rate of aggregation of teratoma cells. The cells were suspended in Hanks' solution containing the indicated concentrations of the following compounds: △, D-glucosamine; ○, D-mannosamine; I, L-glutamine. The range of duplicate values is given for the latter (see text). Assays were conducted after 60-min rotation under standard conditions. All values are relative to that obtained with L-glutamine at 6.9 mM; 10% of the single cells aggregated in this case.

mine, was required for aggregation when the components of Medium 199 were added individually or in combination to Hanks' solution. (3) Omission of L-glutamine from Medium 199 prevented aggregation. (4) Aggregation in Hanks' solution supplemented with L-glutamine proceeded somewhat more rapidly than in Medium 199. (5) The rate of aggregation in Hanks' solution could be increased about 30 per cent when the concentration of L-glutamine was increased from 0.69 to 6.9 mM; the concentration of L-glutamine in Medium 199 is 0.69 mM. (6) Cells suspended in Hanks' solution appeared fully viable for at least three hours at 37°; when L-glutamine was added at any point during this period, aggregation was initiated at a rate comparable to that obtained when the amino acid was added at zero time. (7) Microscopically detectable aggregates appeared in those suspensions where aggregation was indicated by the Coulter Counter method. (8) Of a variety of compounds tested, including the components of Medium 199, only D-glucosamine or D-mannosamine effectively replaced L-glutamine. As observed with L-glutamine, the hexosamines were inactive at 25°. The following compounds did not replace L-glutamine when tested at 6.9 mM concentration in Hanks' solution at 37°: L-glutamic acid (± ammonia), L-asparagine, D-galactosamine, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, N-acetyl-D-galactosamine, N-acetylneuraminic acid, L-fucose, D-galactose, D-mannose, and ethanolamine. (9) Initial rates of aggrega-

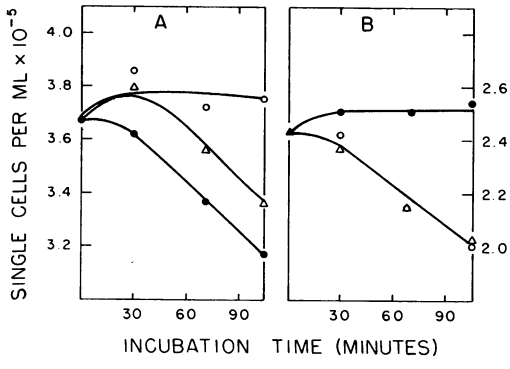

FIG. 4.—Effect of 6-diazo-5-oxonorleucine (DON) on aggregation of teratoma cells. The cells were suspended in Hanks' solution containing the following compounds, and aggregation determined as described in the text. *Expt. A*: O, 3.5 mM L-glutamine and 1.5 mM DON; Δ, 6.9 mM D-glucosamine; ●, 3.5 mM L-glutamine, 1.5 mM DON, and 6.9 mM D-glucosamine. *Expt. B*: ●, 3.5 mM L-glutamine and 1.5 mM DON; Δ, 6.9 mM D-mannosamine; O, 3.5 mM L-glutamine, 1.5 mM DON, and 6.9 mM D-mannosamine. In both experiments, cells were preincubated for 30 min at 37° without rotation in Hanks' solution (with or without DON) prior to the addition of L-glutamine and/or the hexosamines.

tion were approximately equal in Hanks' solution supplemented with either L-glutamine or the hexosamines. However, aggregation continued for somewhat longer periods in L-glutamine than in media containing the hexosamines; this effect may result from the known toxic properties of the amino sugars.[14] (10) A substantial lag period prior to aggregation was noted in some experiments (Figs. 2, 4). We assume that a lag phase occurred in all cases but was not detected in some experiments because of lack of sensitivity of the method, particularly at early time points. (11) The response to L-glutamine and the two hexosamines was completely inhibited by each of the following: NaCN (2 mM), NaN_3 (4 mM), NaF (2 mM), and dinitrophenol (2.5 mM). These inhibitory effects did not result from cell death since the Coulter Counter distinguishes between viable and "leaky" cells. In addition, one inhibitor, fluoride, was examined in detail and the inhibition found to be reversible. Cells, incubated under standard conditions for two hours in Hanks' solution with 6.9 mM L-glutamine and 2 mM NaF, did not aggregate; after a washing (by centrifugation) and resuspension in Hanks' solution without fluoride, the treated cells behaved identically to untreated cells in the presence and absence of L-glutamine. (12) Two metabolic antagonists of L-glutamine blocked the action of the amino acid, but did not inhibit the effect of D-glucosamine or D-mannosamine. The inhibitors, 6-diazo-5-oxonorleucine (DON) and O-diazoacetyl-serine (azaserine), were kindly provided by Drs. J. Ehrlich and T. Haskell of Parke-Davis Laboratories. The former is generally more effective than the latter as an inhibitor of L-glutamine requiring enzymes,[15] including the enzyme that synthesizes D-glucosamine 6-phosphate,[16] and similar results were obtained in the present studies. Experiments on the effect of DON concentration showed that essentially complete inhibition of aggregation was achieved at 1.5 mM concentration in the presence of 3.5 mM L-glutamine. As shown in Figure 4, 6.9 mM D-glucosamine or D-mannosamine overcame the inhibition by DON.

Discussion.—Cells from 129/J mouse teratoma embryoid bodies did not adhere in a glucose balanced salt solution (Hanks'), but did so in tissue culture Medium

199, a complex mixture of known compounds. The difference in behavior of the teratoma cells in the two media permitted identification of L-glutamine as the active factor in Medium 199.

Three possible explanations for the response of the teratoma cells to L-glutamine are as follows: (1) The amino acid is required for survival of the cells; the maintenance and growth of some cell lines in tissue culture does, in fact, require L-glutamine.[17] (2) Adhesiveness results from interaction of cell surface components with L-glutamine by some process such as binding. (3) Adhesive substances are formed by metabolic reactions in which L-glutamine is utilized.

The last explanation appears most probable on the basis of the following observations: (*a*) All available criteria showed that the cells remained viable in the absence of L-glutamine during the short time periods of these experiments. (*b*) Aggregation occurred at 37° but not at 25° (or at 5°). (*c*) Only two compounds were found to replace L-glutamine, i.e., the hexosamines D-glucosamine and D-mannosamine, despite the known toxicity of these compounds to cells maintained *in vitro*. Structural analogues of the active compounds were inactive. (*d*) Metabolic inhibitors blocked the action of L-glutamine and the hexosamines. (*e*) 6-diazo-5-oxonorleucine and azaserine, specific antagonists of L-glutamine, inhibited the action of the amino acid but not of the hexosamines. (*f*) A preliminary experiment[18] showed that labeled D-glucosamine was incorporated by teratoma cells into macromolecules.

L-glutamine is utilized in a variety of metabolic reactions,[15] but the activity of the two hexosamines in promoting aggregation and all the other results presented in this report are consistent with the following hypothesis: The teratoma cells require L-glutamine for the synthesis of amino sugars, and these sugars are utilized for the formation of cell surface macromolecules involved in intercellular adhesion.

Conceivably, only N-acetyl-D-glucosamine residues need be added to the termini of pre-existing oligosaccharide chains on the cell surface to convert nonadhesive to adhesive cells. Indeed, certain transformed cells are agglutinated by a plant lectin that specifically reacts with β-N-acetyl-D-glucosaminopyranosyl groups.[19] However, the known pathways of carbohydrate metabolism,[20–23] which will not be reviewed here in detail, suggest that the process may be much more complex. The relevant reactions in the synthesis of amino sugar containing macromolecules are outlined in Figure 5. These reactions illustrate three major points: (1) Under the usual conditions, D-glucose is the precursor of all nitrogen-containing sugars, the key intermediate being D-glucosamine-6-P. The latter is synthesized from D-fructose-6-P and L-glutamine by a transamidase that is inhibited by 6-diazo-5-oxonorleucine and azaserine.[16] (2) When cells are provided with D-glucosamine, it is converted to D-glucosamine-6-P by hexokinase[24, 25] and then converted to the other amino sugars.[26] Hexokinase also phosphorylates D-mannosamine,[27] but is inactive with D-galactosamine and the N-acetylhexosamines. (3) The oligosaccharide chains of glycoproteins and glycolipids are biosynthesized by the stepwise addition of monosaccharides in a definite sequence.[23] The inability of a cell to synthesize one monosaccharide, such as N-acetylglucosamine, will terminate the process of chain elongation.

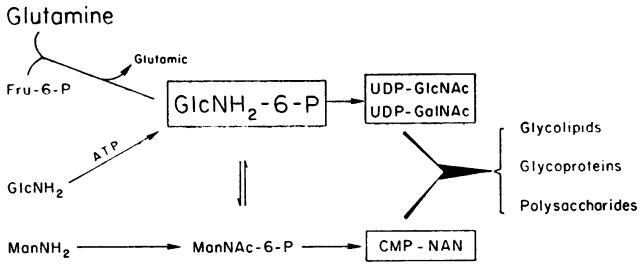

FIG. 5.—Partial pathway for the synthesis of glycolipids, glycoproteins, and polysaccharides. In cells maintained on D-glucose, the key compound D-glucosamine 6-phosphate ($GlcNH_2$-6-P) is synthesized by a transamidase from L-glutamine and D-fructose-6-P; this transamidase is inhibited by DON and azaserine. Abbreviations used are: $ManNH_2$, D-mannosamine; ManNAc-6-P, N-acetyl-D-mannosamine 6-phosphate; UDP-GlcNAc and UDP-GalNAc, the uridine diphospho derivatives of N-acetyl-D-glucosamine and N-acetyl-D-galactosamine, respectively; CMP-NAN, cytidine-5'-monophospho-N-acetylneuraminic acid (CMP-sialic acid).

The conversion of nonadhesive to adhesive teratoma cells may therefore require the addition of only single sugar residues (N-acetylglucosamine, N-acetylgalactosamine, or sialic acid) to the ends of pre-existing oligosaccharide chains, or the process may require the synthesis of the oligosaccharide units in glycoproteins and/or glycolipids, or even of *de novo* synthesis of these macromolecules (or polysaccharides). Studies are in progress to determine which, if any, of these concepts is correct, and to determine the nature of the cell-surface components required for the observed change in the adhesive properties of the teratoma cells.

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